Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 6412–6414, September 1986
Biophysics

Allosteric interpretation of the measurement of cooperative free energy in cyanomethemoglobin

(hemoglobin mechanism/cooperativity/free energy coupling/protein interactions)

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Communicated by John J. Hopfield, May 19, 1986

ABSTRACT **The experimentally resolved cooperative energies in partially ligated cyanomethemoglobin [F. R. Smith & G. K. Ackers (1985) *Proc. Natl. Acad. Sci. USA* 82, 5347–5351] have been compared with the predictions of an allosteric description of hemoglobin. A pattern of energetics similar to that observed (a "combinatorial switch") arises naturally from such an analysis using parameters in excellent agreement with other determinations. Although the energies for 2 of the 10 ligation states (namely, doubly ligated asymmetric tetramers) differ from the predictions, the remaining 8 of the 10 states exhibit excellent quantitative agreement with an allosteric description. This explains the discrepancy between previous analyses, which had found cyanomethemoglobin to be allosteric, and provides support for the basic allosteric concept that quaternary structure is the primary modifier for ligand affinity in hemoglobin.**

A central premise in the attempt to understand the behavior of biomolecules is that the molecular structure should provide insights and ultimately explanations of the function of the molecule. Hemoglobin has been one of the most thoroughly studied biomolecules in no small measure because its structure was one of the first to be solved. Probably the most extensively studied characteristic of hemoglobin function is the cooperativity between binding sites, whereby the binding energy for the fourth oxygen molecule is 3–4 kcal/mol greater than that for the first oxygen molecule.

Of the many attempts to explain cooperativity, the Monod–Wyman–Changeux (MWC) or allosteric model (1) has had the greatest longevity precisely because it permits structure–function correlations. In the allosteric model, the initial and final oxygen binding affinities are characteristics of alternative (R and T) structures of the molecule as a whole. Each of the two structures has a unique binding affinity, and cooperativity is accomplished by the molecular change of structure. This simple model has been elaborated to include phenomenological extensions for inequivalence of subunits (2) as well as stereochemically motivated statistical mechanical interpretations (3–5).

Since its inception, it has been recognized that the allosteric model could not fit all of the available data within the precision with which the data had been gathered. Much of its power lay in the organizing principle that quaternary structure was the principal modifier of affinity, allowing basic thermodynamic questions of cooperativity to be formulated in structural terms. Accordingly, the model retains value to the extent that the primary (though perhaps not the sole) determinant of affinity is an effectively global state of the molecule (the quaternary structure), rather than the state of ligation of neighboring subunits, which is effectively local.

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Smith and Ackers recently reported an ingenious set of experiments in which they measure the interaction energies related to cooperativity (6). Rather than following the ligation procedure directly, which yields too few intermediates for effective study, they formed cyanomethemoglobin intermediates by oxidizing selective subunits to which CN^- was then bound, and then determined the equilibrium constant for dissociation of these partially ligated molecules. Since cooperative hemoglobin tetramers dissociate into noncooperative dimers, the energy of tetramer assembly contains cooperative terms. The variation in associative energy with increasing number of ligands must arise, therefore, from the variation of cooperative energy. In their study, Smith and Ackers found that all intermediates possessed approximately either 3 or 6 kcal of cooperative energy per mol. They concluded that this behavior "contrasts sharply with the premises of a simple two-state MWC model."

There has been considerable experimentation with valency-hybrid hemoglobins, and the literature has twice been reviewed. Shulman *et al.* found the behavior of such valency-hybrid molecules consistent with the basic allosteric description (7). Using a more general statistical mechanical model, Szabo and Karplus similarly found the behavior of the valency hybrids to be allosteric, and they concluded that "in a given quaternary structure, changes in tertiary structure of one subunit do not directly induce significant alterations in the tertiary structure of the other subunits," in support of the central allosteric premise (8). The purpose of the present communication is to examine the data of Smith and Ackers from an allosteric viewpoint to discover how and where this new data departs significantly from an MWC model and, thus, to understand the nature of the disagreement between the work of Smith and Ackers and previous analyses.

ANALYSIS

The diagram in Fig. 1 illustrates the energetics of a basic allosteric model including noncooperative dimers as described by Shulman, Hopfield, and Ogawa (7). Free energy is shown in the vertical direction for each state of ligation of the tetramer. Within a given quaternary structure, there is a unique subunit affinity, hence, the energy levels are equally spaced. The ligand binding energy in the T structure is $\Delta G_T = RT \ln K_T$; ΔG_R is similarly defined for the R structure. The essential manifestation of cooperativity (c) is embodied in the difference in binding energies $\Delta G_c \equiv \Delta G_R - \Delta G_T \equiv -RT \ln c$. Finally, the energy difference between the unligated states is denoted by $\Delta G_L = -RT \ln L$. Although the dimer binds only two ligands, four states (for two dimers) are shown for comparison with the tetrameric states. The ligand binding energy of the dimers is taken to be the same as that of the R state, which ignores the small effect of "quaternary enhancement" (9).

Abbreviation: MWC model, Monod–Wyman–Changeux model.

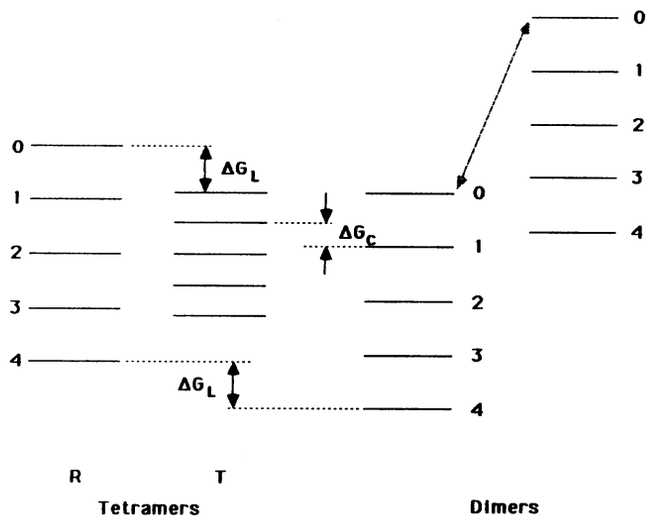

FIG. 1. Free energy diagram for tetramer (R and T structures) and dimer states. The dimer states have been shifted so that the unligated T state and the unligated dimer states are equal to illustrate the subtraction performed by Smith and Ackers (6). The method of Smith and Ackers measures the energy difference between the shifted dimer state and the lowest tetramer state for a given number of ligands. In the diagram, the tetramer R structure and the dimer states are assumed to have the same ligand binding affinity and, hence, the same level spacing—i.e., there is no "quaternary enhancement" (9).

For each degree of ligation, Smith and Ackers calculate the equilibrium constant between tetramer and dimer states. This entails a measurement of the dissociation rate and the assumption that the association rates are all the same. Hence, the energy difference between the tetrameric state and the dimeric state can be computed. The dissociation of unliganded tetramers to dimers requires the energy defined by Smith and Ackers as ${}^0\Delta G_{21}$, which is subtracted from the energy difference between dimer and tetramer for the i-liganded species. Conceptually, this is equivalent to comparing the energy differences under conditions where the deoxy tetramers had the same energy as deoxy dimers, which in practice would be extraordinarily dilute. To illustrate the calculation, the dimers are shown as shifted accordingly in Fig. 1.

In the allosteric model, R and T states are in equilibrium; hence, if one assumes that the equilibrium is maintained, it is necessary to compute the total weighted energy difference from the dimer to the tetramer state—i.e.,

$$\begin{aligned}\Delta G_i &= (\Delta G_{Ri}[R_i] + \Delta G_{Ti}[T_i])/([R_i] + [T_i]) \\ &= (\Delta G_{Ri} + \Delta G_{Ti} L c^i)/(1 + L c^i) \\ &= (\Delta G_L - i\Delta G_c L c^i)/(1 + L c^i),\end{aligned}$$

where ΔG_{Ri} is defined as the energy difference between the R state and the dimers with i ligands, and similarly for ΔG_{Ti}. (This expression omits a small free energy difference arising from the entropy of mixing.) However, given that L is normally quite large, and c, rather small, only one or two ligation states are likely to require both ΔG_{Ri} and ΔG_{Ti} terms. The four-liganded state will be almost purely an R structure, and the energy difference of 5.9 kcal/mol measured by Smith and Ackers for the four-liganded state must be ΔG_L. Thus L must be approximately 2.5×10^4. The singly ligated species is almost a pure T state and the dimer–tetramer energy difference, which is found to be approximately 3.0 kcal/mol (2.9 and 3.2 kcal/mol), will yield ΔG_c. Thus, $c = 0.0053$. In this analysis then, it turns out that ΔG_L approximates $-2\Delta G_c$ (i.e., $Lc^2 \approx 1$). This means that the R and T state energies (and populations) are equal with two ligands bound, and properties of the doubly ligated state will represent an equal mixture of the two states. These parameters validate the assumptions above: the singly ligated species will be in the T structure as assumed, and the triply and quadruply ligated species will be in the R structure. The expected energy difference for the triligated state is $\Delta G_L = 5.9$ kcal/mol; the measured values are 5.8 and 6.0 kcal/mol. The energy difference for the doubly ligated state will be a combination of R and T structure contributions, although both have practically the same energy since $2\Delta G_c$ approximates $-\Delta G_L$. Therefore, the doubly ligated state is predicted to have an energy difference of 6.0 kcal/mol. Of the four possible combinations of ligated and unligated α and β chains that give the doubly ligated state, two yield results (6.2 and 5.9 kcal/mol) close to the expected value. Only 2 of the 10 states behave in an unexpected fashion. These discrepancies will be discussed below.

Table 1 summarizes these results. The magnitude of L and c, and the fact that $Lc^2 = 1$, yields a pattern of cooperative energies with essentially two values. There is, of course, no *a priori* reason for R and T state energies to be equal when two ligands are bound; however, it is interesting to note that the original MWC parameters also had $Lc^2 = 1$ (1). The agreement between the allosteric predictions and the measurements in Table 1 is quite striking. For 8 of the 10 states, the measurements agree within 0.2 kcal/mol (out of 3–6 kcal/mol) with the predicted values; in fact, Smith and Ackers cite 0.2 kcal/mol as the accuracy of their measurement.

The values of L and c used here correspond well with other determinations of those parameters. Ackers and Johnson (10) recently used an allosteric model to analyze the data of Mills *et al.* (11) and obtained for a "typical data set" (see table 2 of ref. 10) $\Delta G_L = 6.3$ kcal/mol and $\Delta G_c = 3.0$ kcal/mol, in good agreement with the values of Smith and Ackers as interpreted above. In their analysis Ackers and Johnson also

Table 1. Comparison of prediction and experiment

Ligation state*		Cooperative free energy, kcal/mol: Measured†	Calculated‡
0	$\alpha^1\ \beta^2$ / $\beta^1\ \alpha^2$	0	0
1	x /	2.9	3.05
	/ x	3.2	3.05
2	x / x	3.0	6.0
	x x /	2.7	6.0
	x / x	6.2	6.0
	x / x	5.9	6.0
3	x x / x	5.8	5.9
	x / x x	6.0	5.9
4	x x / x x	5.9	5.9

*Notation is that of Smith and Ackers (6).
†From ref. 6.
‡Calculated by using $L = 2.4 \times 10^4$ and $c = 0.0053$.

considered the possibility that the dimer binding affinity for ligands is not equal to the R-state binding affinity; in such a model, the difference in binding energy was found to be 0.35 kcal/mol for each ligand. This effect, called quaternary enhancement (9), can be incorporated into the analysis of the data of Smith and Ackers by directly using the value of 0.35 kcal/mol to adjust the measured R-state binding energies. (Smith and Ackers assumed no quaternary enhancement.) Doing so, the ΔG_L measured by the cyanomethemoglobin/hybrid method becomes 7.4 kcal/mol, and ΔG_c becomes 3.35 kcal/mol. In comparison, Ackers and Johnson binding curve analysis gave ΔG_L = 7.7 kcal/mol and ΔG_c = 3.3 kcal/mol, again in excellent agreement.

DISCUSSION AND CONCLUSIONS

The allosteric model is clearly capable of describing the pattern of energetics that Smith and Ackers call a "combinatorial switch" and that gives rise to three distinct energy levels for the 10 ligation states. This agrees with the findings of Shulman *et al.* (7) and of Szabo and Karplus (8) on the applicability of the allosteric model to other valency-hybrid experiments. For 8 of the 10 measured states, the agreement is essentially perfect. The similarity of the parameters generated from the allosteric analysis, when compared with parameters deduced from binding-curve studies, suggests that the cyanomethemoglobin hybrid molecules are a good model system for other ligands. The quantitative agreement between the cyanomethemoglobin/measurement and allosterically analyzed predictions implies very little direct subunit–subunit interaction for most species in the cyanomethemoglobin/derivative. The major premise of the allosteric model thus appears to be sound and surprisingly accurate.

There is no direct and simple explanation for the two hybrids that fail to fit the allosteric pattern. It is possible to speculate that there has been a breakdown of some assumption of the data analysis, such as the existence of a single value of the dimer–tetramer association rate for all species or the rapid equilibration of the R and T structures. However, the possibility also remains that this doubly ligated hybrid has unique energetics that do not fit the allosteric pattern and that warrant an expansion of the simple model described above. In these two cases, the data taken at face value would suggest substantial direct subunit–subunit interaction. Since the principal violation of the simple allosteric model appears to occur for the asymmetric doubly ligated species, further investigation of that state is especially important. The asymmetric doubly ligated state also holds intrinsic interest because the allosteric change is known to strongly affect the pattern of contacts at the $\alpha_1\beta_2$ interface (12–14); hence, this makes further study even more likely. If the asymmetric doubly ligated species continues to fail to fit the allosteric pattern, this would have important implications for the detailed workings of the molecule.

If one assumes that the behavior of cyanomethemoglobin applies to other ligands, this analysis suggests a simple reason for the success of an allosteric model in describing the properties of hemoglobin (7, 8). The only significant departure from the model occurs in the doubly ligated states. These are only slightly populated in binding-curve studies, and their behavior is not likely to affect the analysis greatly. Moreover, attempts to study the doubly ligated state by preparing intermediates have generally used the symmetric doubly ligated species (7), which are easier to prepare than the asymmetric doubly ligated species. Since these hybrids exhibit essentially perfect allosteric behavior, it is again not surprising that the allosteric model was found completely adequate.

The work of Smith and Ackers also appears to provide an improved estimate for the parameter L. In using an allosteric analysis with quaternary enhancement on binding-curve and dissociation data, Ackers and Johnson (10) studied the data of Mills *et al.* (11), as cited above, and found ΔG_L = 7.7 kcal/mol and ΔG_c = 3.3 kcal/mol. However, Ackers and Johnson also analyzed the data of Mills and Ackers (15) (table 3 in ref. 10) and found ΔG_L = 9.0 kcal/mol and ΔG_c = 3.6 kcal/mol. Given such variation, the cyanomethemoglobin method of Smith and Ackers would appear to present a much more accurate determination of the allosteric constant L than does a binding-curve analysis. On the other hand, the measurement of the ratio of binding affinities for first and last ligands, which approximates c, is a fairly accurate one; and since c appears to vary with the specific ligand (7), the method of Smith and Ackers is not likely to be much better than other methods for obtaining that allosteric parameter.

Finally, the allosteric analysis predicts that it is possible to find more than three distinct free energy levels of the system without an increase in the number of species, if the experiment of Smith and Ackers is conducted on a system with appropriate values of L and c (e.g., $Lc^2 > 1$).

I thank Drs. Soumen Basak, William Eaton, and James Hofrichter, who provided helpful suggestions. This work has been supported by Grant AM30239 from the National Institutes of Health.

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